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Impurity profiling of pharmaceuticals by thin-layer chromatography

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ABSTRACT

Although there is a tendency in current pharmacopoeias for favouring HPLC, thin-layer chromatography (TLC) is still a very popular and frequently used analytical method in the pharmaceutical industry. This paper highlights the possibilities of this method in the different areas of pharmaceutical analysis like in-process and intermediate control, illustrated by impurity testing of active ingredients and final products, as well as its application in pharmaceutical research and development, based on some examples reported mainly in the last five years.

1. Introduction

Impurity profiling is a general term including structure elucidation/identification as well as determination of the impurities of a chemical substance. The significance of this process in pharmaceutical research and development has been emphasized multiple times [1–3] with TLC/HPTLC and other planar chromatographic techniques always being mentioned as a widely used and extremely valuable analytical tool in this field.

Besides efficacy and quality, safety of the drug substances and finished drug products is the most important prerequisite in the pharmaceutical industry. Patients of different age and different stage of illness may need to take drugs for long times: therefore these products must comply with maximal standards of safety and quality. International and National Pharmaceutical Authorities, therefore, request and enforce that pharmaceutical manufacturers, licence holders or drug master file holders strictly comply with all Directives and International Guidelines and Regulations of GMP/GXP.

One of the basic prerequisites is the requirement to ensure low levels of the related substances and impurities or degradation products in drug substances and subsequently in drug products. Several guidelines [4–6] have been issued to define allowed or acceptable levels of these impurities in drug substances and drug products (Tables 1 and 2).

In case of genotoxic impurities [6], i.e. substances which are suspected to potentially damage DNA even at very low level of exposure and may contribute to tumour development, a 1.5 μg/day maximum intake is considered to be an acceptable risk. Therefore the analytical limits for these substances are in the lower ppm level in pharmaceutical substances.

As can be seen from the data above, analytical methods must be on hand for active substances and final products which are suitable to detect and quantify impurities at very low levels, i.e. 0.10% or less. The European (Ph. Eur.) [7] and US (USP) [8] pharmacopoeias mainly describe use of HPLC methods for testing the purity of drug substances and finished products, with a tendency to replace established planar chromatographic methods step-by-step.

However, this approach neglects that in the recent years suppliers of TLC/HPTLC equipment have developed new generations of instruments in order to cope with these increased demands. Selectivity was improved by introducing special developing chambers like automatic multiple development (AMD) and overpressured layer chromatography (OPLC). Recent densitometers now allow quantitative evaluation with precision sometimes comparable to HPLC. Video and photo-documentation systems provide GMP/GLP-conform documentation even in case of semiquantitative TLC methods. In addition, in recent years hyphenated techniques have been developed also in TLC like MS-detection for identification and quantitation of impurities.

In spite of new and sophisticated instrumentation, TLC still maintains its advantageous features as summarized by Sherma in comparison to HPLC [9], and recently by Morlock generally and also in hyphenated techniques [10].

2. Planar chromatography in the pharmaceutical analysis

In the pharmaceutical analysis chromatographic separations are used for widely differing purposes, and consequently need dif-

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Table 1
Thresholds for *impurities* in new drug substances [4].

Maximum daily dose	Reporting threshold*	Identification threshold*	Qualification threshold*
≤2 g/day	0.05%	0.1% or 1.0 mg per day intake (whichever is lower)	0.15% or 1.0 mg per day intake (whichever is lower)
>2 g/day	0.03%	0.05%	0.05%

* Lower threshold can be appropriate if the impurity is unusually toxic.

Table 2
Thresholds for *degradation products* in new finished products [5].

Maximum daily dose	Threshold
Reporting threshold	
≤1 g	0.1%
>1 g	0.05%
Identification threshold	
<1 mg	1.0% or 5 μg TDI*, whichever is lower
1 mg–10 mg	0.5% or 20 μg TDI*, whichever is lower
>10 mg–2 g	0.2% or 2 mg TDI*, whichever is lower
>2 g	0.10%
Qualification threshold	
<10 mg	1.0% or 50 μg TDI*, whichever is lower
10 mg–100 mg	0.5% or 200 μg TDI*, whichever is lower
>100 mg–2 g	0.2% or 3 mg TDI*, whichever is lower
>2 g	0.15%

* Total daily intake.

ferent analytical techniques. In case of the final product testing authorities require the maximal accuracy and utmost precision or reproducibility of analytical procedure. The same applies to drug substances, e.g. according to the methods included in pharmacopoeial monographs. As a consequence, usually HPLC is the method of choice for impurity testing of the final products. Nevertheless, previous steps in the manufacturing process allow more freedom for the analyst in choosing the appropriate analytical procedures. It is accepted and reasonable to use any method or techniques, which is precise, rapid, and suitable for the given task. Therefore, TLC/HPTLC or other planar chromatographic separations are frequently used in testing intermediates or for purpose of in-process controls, as well as for purity test of substances in the research and development stage (Table 3).

The wide use of TLC/HPTLC in drug analysis has been reviewed recently by Sherma [11,12] and in a special issue of J. AOAC Int. by Sherma and Krzek as guest-editors [13].

The authors of this paper have summarised the possibilities of TLC in pharmaceutical analysis in previous publications [14–17]; therefore in this paper mainly results of activities in the last five years are presented. Identity test according to pharmacopoeias and the assays of drug product are not covered by this paper. We deal only with purity tests and the impurity profiling applications performed by using different planar chromatographic techniques.

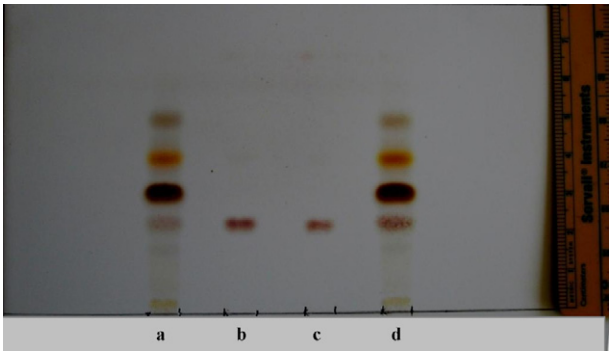


Fig. 1. Limit test of a steroid synthesis in-process control. Applications: (a) reaction mixture corresponding to about 100 μg of the product; (b) 2 μg of the starting material; (c) 1 μg, the limit of starting material; (d) reaction mixture spiked with 1 μg of starting material. Adsorbent, silica gel; mobile phase, *c*-hexane–ethyl acetate 1:1 (v/v); running distance, 8 cm; visualisation, by spraying with ethanolic sulphuric acid; evaluation, in daylight via visible inspection.

2.1. Planar chromatography as in-process control and test for intermediates

The synthetic pathway of an API usually spans over several manufacturing steps. These steps may be discrete or continuous ones. In the first case intermediates are isolated, characterised and analysed individually. In continuous manufacturing processes the intermediates remaining in the reaction mixture are not isolated and controlled individually, however their presence is checked only in the final step of the synthesis. Nevertheless, in both cases in-process control have to be performed to track the progress of the syntheses of the intermediates. As for all in-process control tests there is a need to be performed rapidly and to deliver appropriate information to decide whether the reaction could be stopped or not. Usually this is a simple chromatographic task: starting material and the reaction product should be separated sufficiently to track reaction progress. Examples of this simple task are rarely published in the scientific literature, as this is an exercise usually not justifying a publication however this is one of the most important applications of TLC. In Fig. 1 an in-process TLC-test of a steroid synthesis is presented [18]. This method does not only provide information on the amount of unreacted starting material, but also about the levels of by-products.

Table 3
Use of TLC/HPTLC in pharmaceutical analysis.

Chromatographic test	TLC/HPTLC tests			
	Qualitative	Limit-test	Semiquantitative	Quantitative
Identity test	+			
In-process control		+		
Purity test of				
Intermediate			+	+
Active substance			+	+
Final product			+	+
Assay				+
Dissolution test				(+)
Impurity profiling	+		+	+

The most complex media for synthesis are fermentation broths. When testing fermentation broths using HPLC, the HPLC column can easily suffer irreversible adhesion of the various substances present in the broth and its efficiency may rapidly decrease. This is the reason why planar chromatographic techniques, using disposable sorbent layers, are frequently and successfully applied in checking fermentation processes.

A simple TLC-test was reported for monitoring a fermentation process by Szabó et al. [19]. They followed the formation and degradation of a carbohydrate from starch to a monosaccharide on the same chromatographic plate. The clear supernatant obtained by dilution and centrifugation of the fermentation broth was simply chromatographed on silica gel layer using a chloroform–carbon tetrachloride–35% (v/v) formic acid–methanol 20:5:17:22 (v/v) mobile phase. A sulphuric acidic mixture was used for visualisation by immersion technique, but a densitometric evaluation was also possible prior to the staining.

In a similar way, the bioconversion of soysterols forming androst-4-ene-3,17-dione (AD) and adrosta-1,4-diene-3,17-dione (ADD) was monitored by a simple TLC-method with quantitative evaluation by image-analysis [20]. The bioconversion was tracked by TLC on silica gel sorbent layer using benzene–ethyl acetate 5:1 (v/v) as mobile phase. A sulphuric acidic reagent containing 2% ceric ammonium sulphate was used for visualisation. Authors compared TLC-results and validation characteristics with those obtained by HPLC and found a fairly good correlation.

For improving selectivity, Tamburini and Bernardi used OPLC [21] and AMD [22] methods for monitoring fermentation processes. OPLC is a forced flow planar chromatographic technique [23–25]. In the closed system the vapour phase is eliminated, and the eluent is delivered by forced flow using a pump system, similar to HPLC, enabling constant and optimal flow velocity. This is the reason why it is possible to realise otherwise impossible 18 cm development distances on HPTLC sorbent layer, with the option for further increasing the running distance by overrunning or repeated developments without the loss of efficiency of separation. The authors applied OPLC with overrunning by using acetone–acetonitrile 85:15 (v/v) eluent and densitometric evaluation. The OPLC conditions were as follows: external pressure: 50 bar, mobile phase flow rate, 300 μl min^{-1}, mobile phase volume, 10,000 μl, and a rapid volume of 300 μl. The chromatograms were visualised by dipping in lead (IV) acetate–dichlorofluorescein reagent and subsequently scanned at 313 nm by fluorodensitometry. The method was validated for linearity, and LOD/LOQ, including tests for matrix-effects. Fig. 2 shows the chromatograms of the standard mixture and a fermentation sample.

The authors also repeated the testing of fermentation using AMD-technique [22]. In this system the consecutive developments of the chromatogram are performed by capillary forces in the same direction with equal or usually increasing running distances using stepwise gradient elution with decreasing eluent strength. Between each run the chromatogram is dried. The development process begins with the eluent of the strongest elution power, and then it continues using weaker and weaker eluents according to the desired gradient (10–25 steps). The zone focusing effect of this gradient results in a highly efficient and selective separation [26,27]. A carbohydrate mixture was separated on diol sorbent layer by using a 15-step AMD gradient. The chromatogram was evaluated by derivatisation with aminobenzoic reagent by dipping technique followed by scanning in fluorescence mode (Fig. 3). The authors reported linearity as well as LOD/LOQ data for eight oligosaccharides for both OPLC and AMD techniques.

The impurity profile of an active pharmaceutical ingredient (API) is highly influenced by the quality of its synthetic intermediates. Most of the process related impurities or their precursors in previous steps of the syntheses can contaminate the API [28].

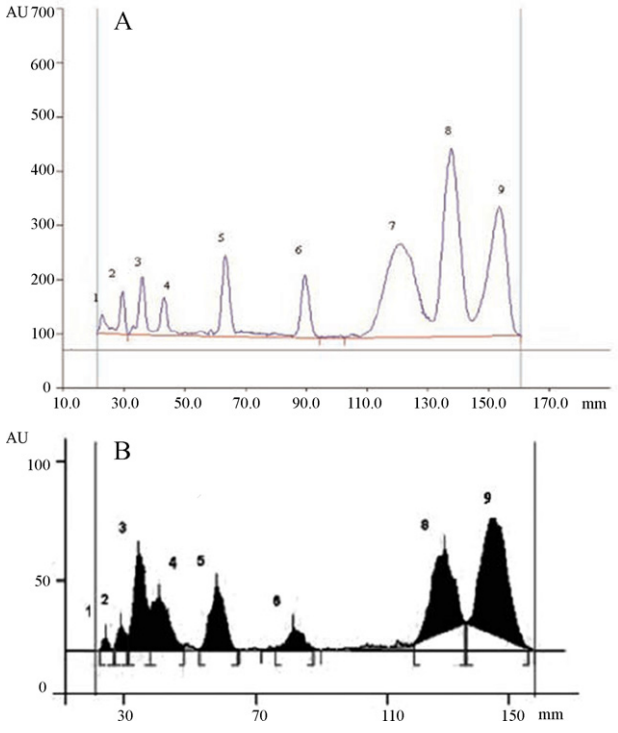


Fig. 2. OPLC chromatogram of standard mixture (A) and fermentation mixture (B), after fermentation by *B. adolescentis* for 5 h: (1), fructosil-nystose; (2), nystose; (3), raffinose; (4), 1-kestose; (5), sucrose; (6), lactose; (7), glucose; (8), galactose; (9), fructose (6 μl of solution diluted 1:100). For chromatographic conditions see text. (Reproduced with permission from [21].)

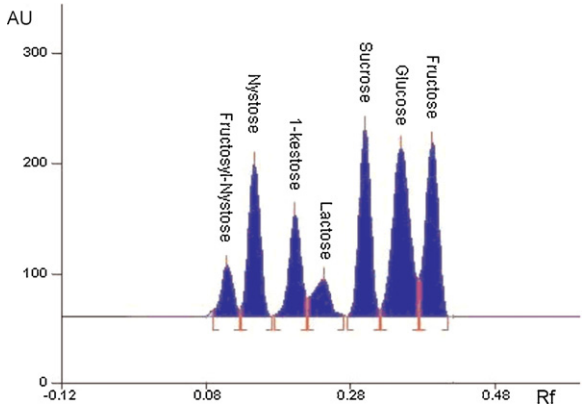


Fig. 3. AMD chromatogram of a sugar-rich solution after fermentation by *B. adolescentis* for 5 h. For chromatographic conditions see text. (Reproduced with permission from [22].)

The prerequisites of a final substance of highest quality standards are intermediate substances of appropriate good quality, so a strict intermediate control is essential. In this stage of the process, analysts are not restricted by the pharmacopoeial monographs' presriptions. So beside "ubiquitous" HPLC, any other technique, like planar chromatographic methods, suitable for the analytical limit to be controlled may be used. Different test methods are particularly important in case of key-intermediates used in different synthesis, influencing the quality of more than one final drug substance. Such a key-intermediate in the nor-steroid total syntheses is nandrolone (19-nortestosterone) which was tested by using a semiquantitative OPLC method [29]. This method was referred to in a previous review on TLC testing the purity of pharmaceuticals [17].

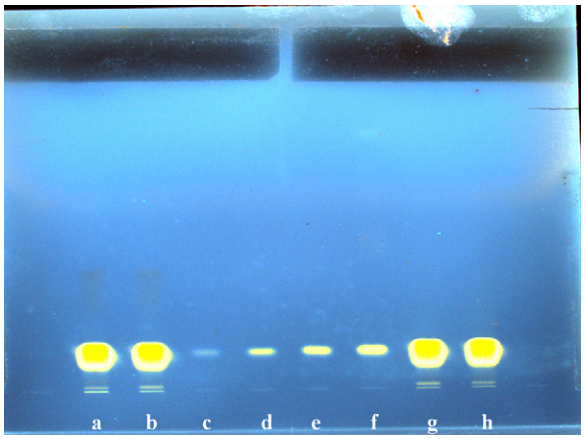


Fig. 4. Semiquantitative purity test of a steroid API according to USP 32. Adsorbent, silica gel; mobile phase, cyclohexane–diethyl ether 4:1 (v/v); applications, 200 μg of samples (a, b, g, h), 0.2, 1.0, 2.0, 4.0 μg of the main component as calibration sequence (c, d, e, f, respectively) running distance, 15 cm; visualisation by ethanolic sulphuric acid reagent; detection at 366 nm, evaluation by visual inspection.

2.2. Testing active substances by semiquantitative TLC

2.2.1. Impurity testing in pharmacopoeias

Although quantitative TLC is described in the general monographs of the Ph. Eur. [7] and USP [8], the individual substance or drug monographs contain only semiquantitative TLC methods. Visual estimation is applied for the decision, whether the tested sample conforms to the impurity limits, as shown in Fig. 4 [30]. The current USP contains a general monograph ⟨466⟩ on "Ordinary impurities" that describes testing by semiquantitative TLC [8a] using different detection modes for visualisation; in this case the general limit for the sum of the impurities is set to 2.0%. This ordinary impurities test is still included or referred in different monographs, but today the authorities will no longer accept this "high" impurity limit for newly submitted synthetic drug substances.

As already mentioned, most of the current purity tests are based on quantitative HPLC or rarely GC, because of the high precision demanded for the control of relatively low limits of related substances. These HPLC methods predominantly use variable wavelength or diode array UV detectors; MS-detectors are rarely used in the routine QC laboratories. As a consequence, substances having low UV-absorbance cannot be detected by this mode. GC methods, on the other hand, are nearly exclusively used for determination of the residual solvents of the drug substances, mainly because the drug substances are usually non-volatile and easily undergo heat degradation during the GC-analysis, like nandrolone [29]. For that reason, TLC is the purity test of choice also in some pharmacopoeial monographs. As an example, *pancuronium bromide*, a quaternary ammonium salt having no UV-activity, can only be chromatographed by ion-pair technique, using sodium iodide 400 g/l–acetonitrile–2-propanol 5:10:85 (v/v) as eluent. Aggressive ion pair reagents may cause corrosion in the highly expensive HPLC-equipment, therefore it is tested by a TLC method using disposable silica gel sorbent layer using Dragendorff's reagent for visualisation [7a].

In some cases TLC is a supplementary purity test to HPLC, as in the case of *nandrolone decanoate* [7b]. According to the current Ph. Eur. monograph some impurities of nandrolone decanoate are to be tested by HPLC, but three of them, showing no UV-activity, can only be tested by TLC after sulphuric acidic visualisation and semiquantitative evaluation. Fig. 5 shows a TLC chromatogram of nandrolon decanoate before and after the sulphuric acidic visualisation. Similarly, combined or supplementary HPLC and TLC purity tests are applied for testing substances containing impurities which can be detected in HPLC only with difficulties, like: *altizide* [7c], *perindopril* [7d], *testosterone* [7e], *zidovudine* [7f], or *topiramate* in USP [8b]. The Japanese Pharmacopoeia [31] prescribes more TLC purity tests than Ph. Eur. and USP.

A very unique area of pharmaceutical manufacturing is the synthesis of radiopharmaceutical preparations. TLC is often used for testing radiochemical purity of the preparation according to the European Pharmacopoeia, by using special detectors for evaluation of the chromatograms [7g].

2.2.2. Investigation of 19-norsteriods by OPLC method

The total synthesis of 19-norsteriods consists of many steps and results in different drug substances including some intermediates exhibiting low UV-absorbance. Therefore planar chromatographic methods may be well used for investigation of these substances. Monographs in previous editions of pharmacopoeias and in case of levonorgestrel/norgestrel even the current ones include thin

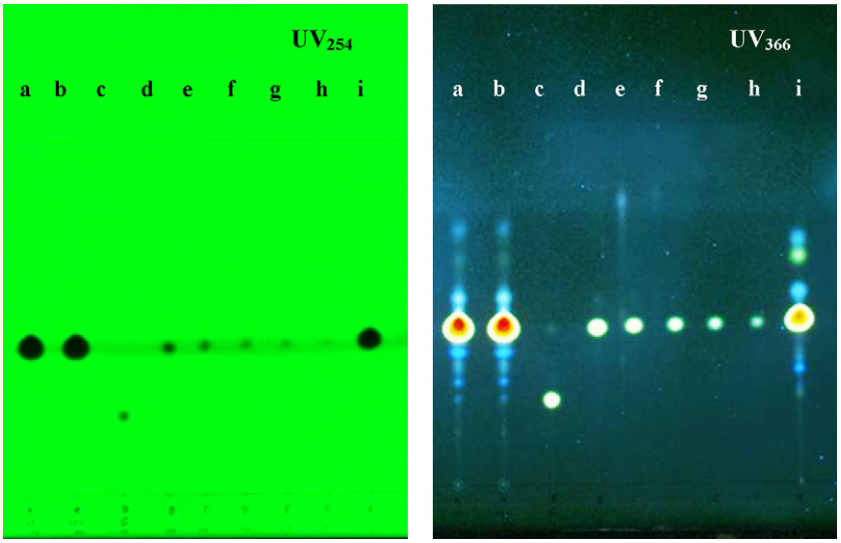
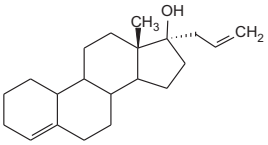
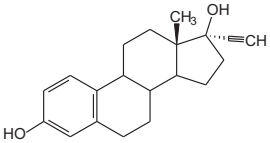
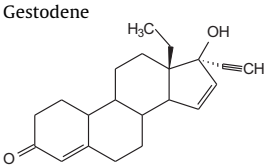
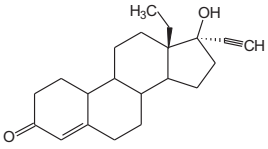
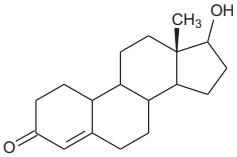
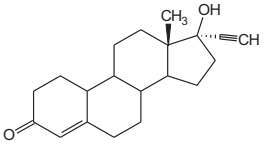


Fig. 5. Semiquantitative purity test of nandrolone decanoate for impurities A, B, C according to Ph. Eur. Applications, (a) and (b) 100 μg of samples; (c) 0.5 μg of nandrolone; (d)–(h) 2.0, 1.0, 0.5, 0.3, 0.1 μg of the main component as calibration sequence; (i) 100 μg of system suitability CRS containing impurities A, B, C; detection at 254 nm and after sulphuric acidic visualisation at 366 nm. Adsorbent, silica gel; mobile phase, acetone–heptane 30:70 (v/v).

Table 4
OPLC methods of 19-norsteroids.

Name and structure	OPLC method (chamber: P-OPLC BS 50, external pressure: 50 bar, sorbent layer: sealed HPTLC silica gel)					
	Eluent	Developing eluent volume (μl)	Developing mode	Flow rate (μl min^{-1})	Visualisation	Ref.
Allylestrenol 	cyclohexane–butyl acetate–chloroform (90:12:2)	6500	Overrunning	300	Sulphuric acid	[34]
Ethinylestradiol 	cyclohexane–ethyl acetate–chloroform (3:1:1)	7000	Overrunning	300	Sulphuric acid	[17]
Gestodene 	cyclohexane–ethyl acetate–chloroform* (3:1:1)	6500	Overrunning	300		[35]
Levonorgestrel Norgestrel 	System A: for impurities $R_{f,rel}$ < 1 Toluene–ethyl acetate–chloroform (50:10:40) System B: for impurities $R_{f,rel}$ > 1 Cyclohexane–butyl acetate–chloroform (60:20:20)	S_A: 1000 2000 3000 4000 S_B: 4200	S_A: multiple S_B: single	400	Phosphomolybdic acid or Sulphuric acid	[32]**
Nandrolone 	Cyclohexane ethyl acetate–chloroform (50:25:25)	2000 3000 4000	Multiple	300	Sulphuric acid	[29]
Norethisterone 	1: *n*-Hexane; 2: butyl acetate–chloroform (85:15)	4000 + 4000	Continuous, overrunning	400	Sulphuric acid	[33]

* Ethanol-free.
** Without mentioning the name of the substances tested.

layer chromatographic purity tests with acidic spray-reagents for visualisation. The advantage of these methods is that all the impurities, including those showing no UV-activity, can be detected on the chromatographic plate however these methods will not separate the closely related steroid impurities from each other and from the main component. By using OPLC [23–25] selective separation of these substances could be achieved. The OPLC helped to identify the impurity profiles of *ethinylestradiol* [17], *levonorgestrel* and *norgestrel* [32], *norethisterone* [33], *allylestrenol* [34], *gestodene* [35] and the key-intermediate *nandrolone* [29]. These steroid molecules, originating from the ethyl and methyl-series of norsteroid total syntheses, have some similar derivatives, as well as special impurities characteristic only for one individual substance. Because the retardation factor (R_f) is very well reproducible in OPLC, based on the relative retardation factors ($R_{f,rel}$), the similar impurities can be arranged in a polarity series, which also can help to predict the structure of related substances of new steroid substances of this family. Table 4 summarises the parameters of the OPLC purity test methods of some known norsteroids, whereas Table 5 summarises similar derivatives present in the different norsteroids according to their polarity, according to Bagócsi [36].

Although recently HPLC methods have been developed for purity testing of these steroids, the OPLC methods still play an

Table 5
Polarity series of similar derivatives of 19-norsteriods [25].

Polarity series (decreasing order)	Allylestrenol [34]	Ethinylestradiol [17]	Gestodene [35]	Levonorgestrel norgestrel [32]	Norethisterone [33]	$R_{f,rel}$
6β-OH	–	+ (Imp. F)	+ (Imp. D)	+	+ (Imp. H)	0.0–0.3
6α-OH	–	+ (Imp. E)	–	+	+	
10-OH	–	-	–	+	+	
6-keto	–	+	+ (Imp. E)	+	+	0.2–0.5
$\Delta^{8(14)}$	+	–	–	+ (Imp. A)	–	0.8–1.0
Δ^{6}	–	+ (Imp. I)	+ (Imp. A)	+	+ (Imp. A)	
Main component						1.0
17-epi	–	+ (Imp. A)	–	–	+ (Imp. G)	1.0–1.2
Δ^{5}	–	–	+ (Imp. L)	+	+ (Imp. C)	1.1–1.6
$\Delta^{5(10)}$	–	–	+ (Imp. B)	+ (Imp. B)	+ (Imp. D)	

Imp. A–L: according to the Ph. Eur. monographs; +/–: impurities detected/not detected.

important role in recognition of their impurity profile and may be used as an alternative method for fast analysis as well as in cleaning validation procedures [37].

2.3. Quantitative determination of the impurities

Improved precision in TLC analysis is attained by using contemporary quantitative densitometric evaluation. In this way, similarly to HPLC, an objective accurate and precise evaluation can be achieved, and the analytical raw data and the print-out of the chromatograms can be stored according to GMP. Modern densitometers work in wide wavelength ranges of 190–800 nm, allowing to perform the detection at the maximal or optimal absorption wavelength of the substances investigated.

In this way the sensitivity of the TLC/HPTLC methods can be increased significantly. Since several years densitometers equipped with diode-array detector, similarly to HPLC, are also available [38]. Although peak shape of reflexion UV spectra is poorer than that of the spectra recorded in solutions, identity information of the separated substances can be obtained.Maślanka and Krzek [39] developed a quantitative TLC densitometric purity test for some psychotropic drugs – *chlorpromazine hydrochloride*, *trifluoperazine dihydrochloride*, *promazone hydrochloride* and *doxepin hydrochloride* – for which only semiquantitative TLC was described in the pharmacopoeias. The separation was performed on TLC silica gel sorbent layer by using cyclohexane–acetone–diethylamine 8:1:1 (v/v) mobile phase, and propan-2-ol–diethylamine 50:1 (v/v) in case of doxepin HCl. Densitometry was performed at 254 and 290 nm. The impurities could be determined with a sufficient precision of less than 10% RSD.

Mitrazapine, an antidepressant and its three main process-related impurities were separated and determined densitometrically by Reddy and Devi [40]. The separation was performed on HPTLC silica gel sorbent layer with 8 cm running distance by toluene–acetone–methanol 6:2:2 (v/v) as mobile phase. Evaluation was performed at 285 nm, at the absorption maximum of the impurities, in reflection mode (Fig. 6).

In some cases, even when HPLC test is the pharmacopoeial mandatory method, an additional quantitative planar chromatographic method may be developed in parallel allowing rapid and economical impurity analysis, suitable for the purity testing of both API and its dosage form, as well as an assay of the lat-

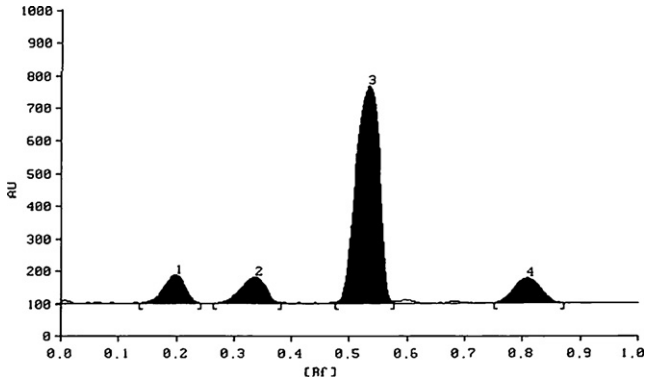


Fig. 6. Densitogram showing the separation of mitrazapine (3) and its impurities (1, 2, 4) using toluene–acetone–methanol 6:2:2 (v/v), scanned at 285 nm. (Reproduced with permission from [40].)

ter. Such a method was published by Agbaba et al. [41]: the antiplatelet agent *clopidogrel* and its hydrolytic degradation product SR 26334 were separated on silica gel sorbent layer by using *n*-heptane–tetrahydrofuran 1:1 (v/v) mobile phase. For quantification the chromatograms were scanned at 230 nm (Fig. 7). The

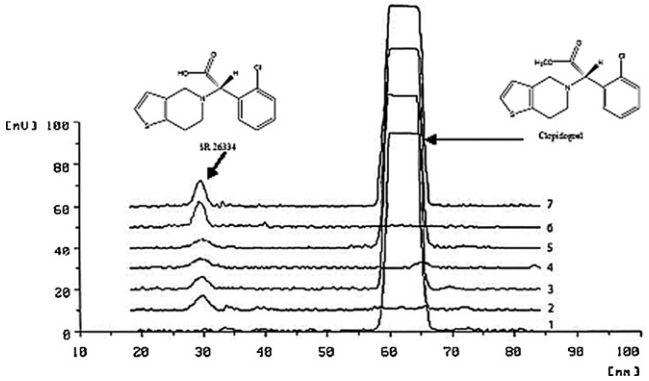


Fig. 7. Densitograms obtained from a reference clopidogrel sample (1); 100, 50, and 200 ng SR26334 standard (2, 4, 6, respectively); and samples of clopidogrel spiked with 0.2, 0.1 and 0.4% SR 26334 (3, 5, 7, respectively). (Reproduced with permission from [41].)

method was also used to assay of clopidogrel in pharmaceutical dosage forms with a 98–103% recovery ($n = 6$), sufficient for quality control of the dosage form.

The same TLC method was used for *impurity testing* of *pantoprazole* and *omeprazole* APIs and in tablets, as well as for *assay* of omeprazole and pantoprazole in tablets [42]. These heterocyclic compounds were separated on HPTLC sorbent layer with chloroform–2-propanol–25% ammonia–acetonitrile 10.8:1.2:0.3:4 (v/v) as mobile phase.

Krzek et al. reported about determination of *ciprofloxacin* and its impurities and degradation products [43]. An HPTLC silica gel layer was used with chloroform–methanol–25% ammonia 43:43:14 (v/v) as mobile phase, and the detection was performed at 277 and 330 nm based on UV-absorbance maxima of the substances investigated.

According to the Ph. Eur., the related substances of *clopamide* are to be determined by a gradient HPLC method [7g]. Krzek et al. [44] developed a simple quantitative densitometric method for determination of these substances in tablets. They used silica sorbent layer with a mobile phase of *n*-butanol–2-propanol–water–methylene chloride–methanol 10:7:2:5:3 (v/v). The evaluation was performed at 235 nm. The analytical results obtained by TLC and HPLC were very similar both in assay and in precision of the method.

The special form of establishing an impurity profile is the forced degradation or stress-testing as described in ICH Guideline Q1A "Stability Testing of New Drug Substances and Products" [45] and referred to ICH Guideline Q3A "Impurities in New Drug Substances" [4]. This approach is the appropriate one in the course of validation of a purity test or an assay of a new entity whose degradation products are yet unknown or not yet characterised. The aim of this investigation is to prove that the method is "stability-indicating" by testing samples artificially degraded by acidic, alkaline, thermal, oxidative and light stresses. These stress-produced impurities rarely arise from the substance during the manufacture or normal storage of the substances. Nevertheless, the stressed degradation products can characterise the substances, and prove the stability indicating nature of the analytical method. However it must be considered that this approach does not take into consideration any impurities arising from synthesis and is intended only for new chemical entities or new drugs with new excipient profile. Nevertheless, there are numerous examples in the analytical literature describing repeated stress testing of long known drug substances with well known degradation and impurity profiles.

An example for stress testing by TLC is the article published by Starek et al. [46] about the determination of heat and pH-induced degradation products in *rofecoxib* drug substance and its formulation. The separation was performed on a silica gel sorbent layer with chloroform–acetone–toluene–glacial acetic acid 12:5:2:0.1 (v/v) mobile phase and evaluated by densitometry at 256 nm. The LOD and LOQ were 0.35–1.05 μg, and the recovery exceeded 98%.

The same team investigated the stability of *piroxicam* by quantitative TLC on silica gel sorbent layer by using ethyl acetate–toluene–butyl amine 2:2:1 (v/v) as mobile phase, with densitometric detection at 360 nm [47]. Two decomposition products induced by acidic media and elevated temperature were identified by ^{1}H NMR and LC–MS–MS method.

A quantitative HPTLC method for *ezetimibe*, a lipid lowering compound was developed by Mahadik and Dhaneshwar [48]. The separation was performed on an HPTLC silica gel layer by using toluene–ethyl acetate 7:3 (v/v) as mobile phase and densitometric detection at 254 nm. They stressed the active substance by 3% and 30% hydrogen peroxide, by neutral aqueous hydrolysis, by sunlight, as well as dry and wet heat. Different degradants were separated as shown in Fig. 8 in case of photo-degradation. The method was applied for an already marketed formulation. A quite similar

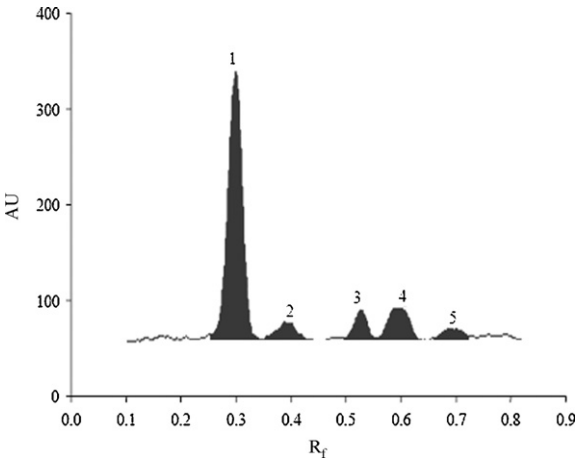


Fig. 8. Densitogram of photo-degraded ezetimibe. Peak 1, ezetimibe, (R_f: 0.31); peaks 2, 3, 4 and 5, degradants (R_f: 0.40, 0.52 0.60 and 0.70). (Reproduced with permission from [48].)

work was published in case of *amtolmetin guacil* in pharmaceutical dosage form [49].

A similar stress-test was applied for proving the stability indicating feature of a quantitative TLC method using for determination of *telmisartan* and *ramipiril* in combined tablet dosage form for the treatment of high blood pressure [50]. The separation was performed on TLC silica gel by using methanol – chloroform 1:6 (v/v) as eluent and the chromatograms were evaluated at 210 nm. The degradation was induced by alkaline and oxidative stresses.

2.4. TLC in the research and development, identification of the unknown impurities

In the development phase of a new pharmaceutical entity, TLC is the most frequently used analytical technique. Initially it is used as a simple way to control the synthesized substance during the reaction sequence. When the synthesis is successful, the final substances are investigated by different analytical methods to gain information about their impurity profile. Some of the impurities can be predicted based on scientific consideration: the last intermediate, as well as some other process-related and drug-related impurities. These are synthesized during the development of the synthesis and identified in the final substance by matching their chromatographic retention parameters compared to those of the impurity spots in the product.

Nevertheless, unknown impurities are usually also found and have to be identified in the research stage. Two types of chromatographic methods, reversed phase HPLC and normal phase TLC together are predominantly used for this purpose. HPLC provides a good selectivity and efficiency, but impurities having significantly different polarities may be invisible even in case of MS-detection, remaining on the column or eluting together with the solvent peaks. In contrast to this, TLC can test a wider polarity range, so all the impurities can be detected on the chromatoplate form the starting point to the eluent front, even those remaining on the origin or those that cannot be separated from each other or from the main component. For this reason an HPLC purity test may only be accepted for validation if its results are in good correlation with the previous TLC-investigations.

An example is a quantitative TLC test method developed for an intermediate of an original active substance in research stage [51]. The general purity test of the API was planned to be performed with a HPLC method, but one of the impurities significantly differed in its structure and polarity from the main components and the other related substances. Therefore a fast quantitative TLC

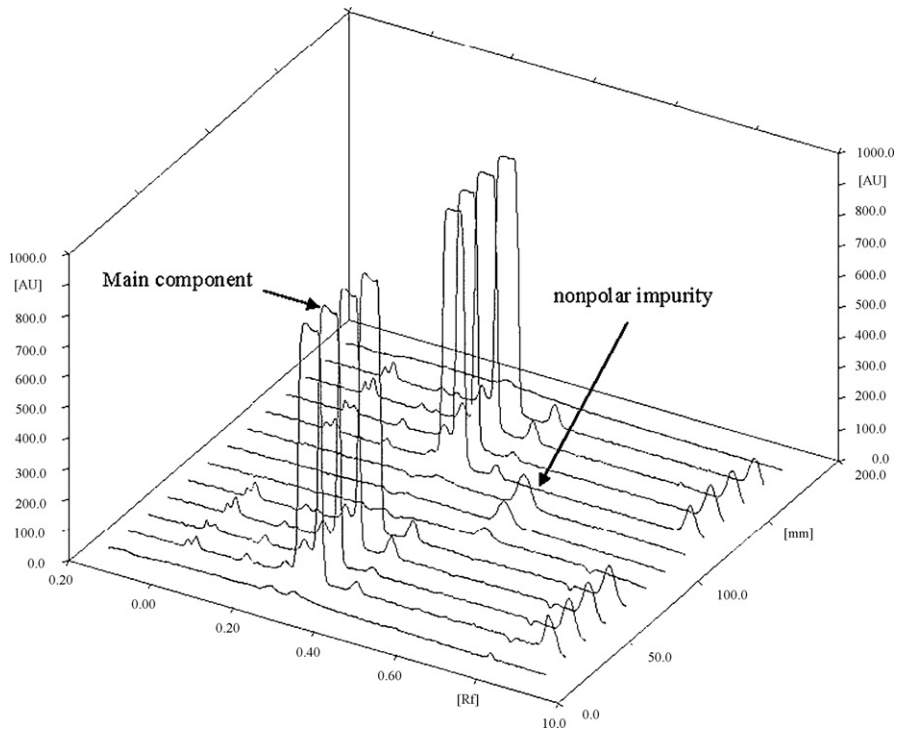


Fig. 9. Quantitative purity test of a highly nonpolar intermediate of an active substance. Adsorbent, silica gel; mobile phase, ethyl acetate–methanol–cc. ammonia 80:10:10 (v/v). Applications, 100 μg of samples, 0.05, 0.10, 0.15 μg of intermediate standard blank; running distance, 8 cm, evaluation by densitometry at 290 nm.

method was developed for testing to avoid the time-consuming development of a difficult gradient HPLC method in this stage of research. The combined results of the TLC and the HPLC method gave satisfactory information about the purity of the substance (Fig. 9).

As mentioned above, to find all the possible degradation products of a new entity, a stress-test as a preliminary stability test is usually performed. A special form of the stress-test is the photostability testing in which the substance under investigation is irradiated with a defined wavelength and intensity of light according to the ICH-guide [52]. The photochemical degradation of *fluvastatin* in solution was tested by Mielcarek et al. [53]. They detected three photo degradation products by reserved phase HPTLC method using silica gel RP-18 F_{254} sorbent layer with phosphate buffer (pH 4 ± 0.05)–methanol 3:17 (v/v) eluent. The development had to be performed at 4 ± 0.5 °C temperature with 9 cm running distance, and visualisation was achieved at 254 nm UV-light. After separation, the degradation products were isolated by scraping the spots from the sorbent layer and extraction with methanol. The degradation products were identified by UV-spectroscopy.

Two unknown impurities of *8-chlorotheophilline* were detected by TLC and HPTLC method by using silica gel sorbent layer and ethyl acetate–hexane 60:40 (v/v) mixture containing 2–3 drops of glacial acetic acid. The unknown impurities were isolated by preparative TLC using home made sorbent layer of 2–5 mm thickness using the same eluent as used in the analytical separation. After separation the unknown impurities were scraped off and extracted with methanol, and finally identified by GC–MS as N-chloromethyl-derivative and hydrated form of N-chloromethyl derivate of 8-chlorotheophylline [54].

Today the identification of the unknown impurities can be achieved far more smartly avoiding the time-consuming isolation by scraping-off followed by MS-determination by using the newly spread hyphenated TLC–MS coupled technique that complements the known HPLC–MS and GC–MS coupling techniques. The most advantageous feature of getting samples directly from the TLC plate is that the individual impurities can be tested one-by-one, spot-by-spot, and that eluent components do not interfere with the MS-detection as they are eliminated after the chromatographic process by drying the sorbent layer. Comprehensive reviews about the different type of TLC–MS coupling were issued by Morlock et al. [10,55,56], who introduced this technique in testing food-stuffs. The application of the TLC–MS technique is currently facilitated by different TLC–MS interfaces commercially available based on desorption electrospray inonisation (DESI) [57] or extraction [58].

Matrix-assisted laser desorption ionisation mass spectrometry (MALDI-MS) directly combined with TLC was critically reviewed by Schiller et al. [59] with examples for application on proteins, peptides, nucleic acids, pharmaceuticals and lipids. This technique was used by Crecelius et al. for pharmaceutical analysis [60] and for identification and quantification of drug substances and their related substances in R&D stage [61].

2.5. Chromatography of optical isomers

Testing APIs for chiral impurities has been also introduced in the pharmacopoeias e.g. in case of *dexchlorpheniramine maleate* or *timolol maleate* in the corresponding Ph. Eur monographs [7h,i]. In every cases the compendial test method is HPLC. However there are also different possibilities for testing optical purity by TLC too, as was comprehensively reviewed in a recent book edited by Kowalska and Sherma [62]. Nevertheless, methods developed for chiral separation by TLC are usually applied to separate of optical isomers being present in similar or commensurable quantities in pharmaceuticals [63–67]. Chiral purity testing based on TLC separation still requires different chiral additives or prechromatographic derivatisation, thus discouraging analysts to use this technique to control optical isomers in the routine quality control applications. In this area, HPTLC loses its advantageous features compared to HPLC which allows separation of the isomers after simple method development by using special chiral columns for this purpose. This

situation could be changed only with commercially available inexpensive precoated chiral plates [68].

2.6. Screening by TLC

The strictly validated analytical methods used in pharmaceutical quality control are developed to detect and determine the impurities *expected* in the APIs or in the drug products. When *unexpected* impurities may appear, e.g. in case of cross-contamination or in testing substances of new or unknown origin, it is highly recommended to use at least two different chromatographic methods for testing and one of these should appropriately be TLC.

As an example, TLC was used for testing an intermediate of methamphetamine, the active component of Ecstasy tablets [69,70]. The impurity profile detected by TLC can be used to identify the origin of the tablet. Authors applied optimised solid phase extraction to isolate the main components and their related substances from the tablet, and tested the effect of the matrix-components on the extraction, because the matrix might also be different and characteristic for the different origins of the tablets.

TLC is also frequently used and widely promoted e.g. by WHO for screening of drug products to detect counterfeit drugs. Mobile mini laboratories like the Global Pharma Health Fund's Minilab® [71] that are operated world-wide use this simple method for a rapid screening, whether a product contains the active substance as labelled or not, or has similar impurity profile as the standard drug product of known and legal origin [72,73].

3. Conclusion

Thin layer chromatography is a widely used method in the pharmaceutical analysis both in its classical semiquantitative form, and equipped with sophisticated analytical instruments like special chamber-types (OPLC, AMD), densitometers, or coupled with different interfaces with MS-spectrometers for identification and quantitative analysis of impurities.

Surveying the publications of the last five years, one has to appreciate the activity of the Polish and Indian experts in this area.

Besides *Journal of Planar Chromatography*, specialized in planar chromatography, and other well-known classical chromatographic or pharmaceutical periodicals like *Journal of Chromatography*, *Journal of Liquid Chromatography and Related Technologies*, *Chromatographia*, *Acta Chromatographica, Journal of Pharmaceutical and Biomedical Analysis* to name a few, recently a number of interesting periodicals have appeared from East like *Current Pharmaceutical Analysis*, *International Journal of Pharmaceutical Sciences Review and Research*, or *Asian Journal of Pharmaceutical Sciences* which are worth to attract the attention of analyst dealing with TLC and/or pharmaceutical analysis.

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